Advances in Brief

Adenovirus 5 Early Region 1A Does Not Induce Expression of the Ewing Sarcoma Fusion Product EWS-FLI1 in Breast and Ovarian Cancer Cell Lines¹

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Abstract

The adenovirus 5 early region 1A (E1A) can function as a tumor suppressor gene and is being used in clinical trials as a therapeutic agent for advanced breast, ovarian, and head and neck cancer. Recently, there has been a dispute regarding whether transfection with the E1A gene can induce expression of the Ewing sarcoma oncogenic fusion transcript EWS-FLI1 (Sanchez-Prieto et al., Nat. Med., 5: 1076–1079, 1999; Melot and Delattre, Nat. Med., 5: 1331, 1999; Kovar et al., Cancer Res., 60: 1557–1560, 2000). In an effort to settle the controversy, we tested several stable E1A transfectants of cell lines MDA-MB-231, MCF-7, MDA-MB-435 (breast cancer), SKOV3-ip1 (ovarian cancer), and PC-3 (prostate cancer), as well as parental and vector-transfected controls, HEK 293 cells, and RD-ES (Ewing sarcoma) cells, for the EWS-FLI1 fusion product. The EWS-FLI1 transcript could not be identified with reverse transcription-PCR in any of the 13 E1A-transfected cell lines analyzed. Furthermore, the EWS-FLI1 fusion protein could not be detected by Western blot analysis in E1A-transfected cell lines. These results suggest that E1A transfection does not necessarily lead to expression of the oncogenic EWS-FLI1 fusion transcript. Thus, the potential induction of this gene rearrangement by E1A gene therapy is unlikely to be clinically significant in the treatment of advanced malignant disease.

Introduction

The adenovirus E1A gene function as a tumor suppressor gene (reviewed in Refs. 1 and 2). E1A can down-regulate the erbB2 oncogene to suppress tumorigenesis, and it can suppress metastasis by additional molecular mechanisms. Furthermore, E1A sensitizes cells to cellular host immune responses and to apoptotic agents such as anticancer drugs, tumor necrosis factor, and irradiation. The E1A protein is under active investigation as a potential therapeutic agent. A Phase I clinical trial of E1A gene therapy for patients with advanced breast and ovarian cancer has recently been completed (3), and Phase II trials are currently in progress.

Ewing sarcoma 11;22 translocation results in a rearrangement between EWS on chromosome 22q12 and FLI1 on chromosome 11q24, leading to a chimeric transcription factor, EWS-FLI1, that has potent transforming activity (4). Sanchez-Prieto et al. (5) have recently reported that the adenoviral E1A protein has the ability to induce expression of the EWS-FLI1 fusion protein. However, data from the studies of Melot and Delattre (6) and Kovar et al. (7, 8) do not support this concept. De Alava et al. (9) have suggested that this discrepancy may be due to the types of subclones and assay conditions used. Because of this controversy and because the induction of the oncogenic EWS-FLI1 transcript by E1A transfection could have significant consequences in the setting of E1A gene therapy, we tested stable, E1A-transfected breast and ovarian cancer cell lines for the expression of the EWS-FLI1 fusion product.

Materials and Methods

Cell Cultures, Plasmids, and Retroviral Vectors. The human breast cancer cell lines MCF-7 and MDA-MB-231 and ovarian cancer cell line SKOV3-ip1 with and without transfection of E1A were routinely maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO). Stable cells expressing wild-type E1A or 12S E1A were established by transfecting cells with the E1A-expressing plasmid pE1A-neo or 12S E1A plasmid DNA plus pSV-neo. The transfectants were grown under the same conditions as controls, except that G418 was added to the culture medium. Expression of E1A was verified by Western blot analysis using monoclonal anti-E1A antibody M58 (Oncogene Science, Cambridge, MA). The human Ewing sarcoma cell line RD-ES was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 15% fetal bovine serum.

¹The abbreviations used are: E1A, 5 early region 1A; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RT-PCR. Total RNA was isolated from cell lines with Trizol reagent (Life Technologies, Inc.). One μg of total RNA was reverse transcribed after priming with oligo(dT) primers using the Superscript Premplification System (Life Technologies, Inc.). The single-stranded cDNA was amplified by 40 PCR cycles of denaturing at 94°C for 45 s, annealing at 58°C for 1 min, and extension at 72°C for 30 s. RNA from the Ewing sarcoma cell line RD-ES was used as a positive control. Primers described previously by Sanchez-Prieto et al. (5) were used: (a) 11.3 FLI1, 5'-ACTCCCCGTGTTGCTGCCCCTCC-3'; (b) 22.3 EWS, 5'-TCTTACAGGCAAGCTCAAGTC-3'; (c) 11.4 FLI1, 5'-CAGGTTGATACAGCTGGCG-3'; and (d) 22.4 EWS, 5'-CCAACAGAGCAGCTAC-3'. Nested PCR was first performed using the 11.3 FLI1 and 22.3 EWS primers and then performed using the 11.4 FLI1 and 22.4 EWS primers. Expression of the E1A transcripts was confirmed by RT-PCR using primers 5'-GGCCTGCTATCCTGAGA-3' and 5'-CCGCTCAGTATGCTGGGCGTACG-3'. Amplification of GAPDH was performed as a control using primers 5'-AAGGTGAAGGTCGGAGTCAAC-3' and 5'-CATGAGTCCTTCACGATACC-3'. Amplified products were analyzed by 1.5% agarose gel electrophoresis.

Cloning and Sequencing. RT-PCR using the 11.4 FLI1 and 22.4 EWS primers (Fig. 1a) and the 11.3 FLI1 and 22.4 EWS primers was performed with RNA from the E1A-transfected MDA-MB-231 cell line and the RD-ES sarcoma cell line. The amplified products were subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were sequenced and compared with GenBank database sequences from the National Center for Biotechnology Information using the BLAST algorithm.

Southern Blot Analysis. RT-PCR products were separated by 1.5% agarose gel electrophoresis and blotted onto nylon membranes (GeneScreen; New England Nuclear Life Science Products, Boston, MA). The membranes were hybridized in RapidHyb buffer (Amersham Corp, Arlington Heights, IL) and probed with the RD-ES 11.4 FLI1/22.4 EWS fusion fragment subcloned previously. The probe was randomly labeled with [32P]dCTP using the random primers DNA labeling system (Life Technologies, Inc.). The 22.4 EWS oligonucleotide was radiolabeled with [γ-32P]ATP with T4 polynucleotide kinase (New England Biolabs, Beverly, MA).

Western Blot Analysis. Western blot analysis of whole cell extracts was accomplished using standard procedures. Briefly, cells were collected and lysed with buffer A [20 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 0.15 M NaCl, 1 mM β-mercaptoethanol, 1 mM Na3VO4, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% (w/v) bromophenol blue]. Samples were heated at 100°C for 5 min and separated by 10% SDS-PAGE. After transfer, membranes were incubated with either the M73 antibody (Onco-gene Sciences, Cambridge, MA) against E1A protein, the antibody against FLI1 (PharMingen, San Diego, CA), or the N-18 antibody against EWS (Santa Cruz Biotechnology, Santa Cruz, CA). As a loading control, membranes were also incubated with an antibody against actin (Boehringer Mannheim, Indianapolis, IN).

Results

EIA-transfected Cell Lines Do Not Express the EWS-FLI1 Fusion Transcript. Stable E1A transfectant of breast cancer cell line MDA-MB-231 (231-EIA), parental and vector-transfected controls, E1A-expressing HEK 293 cells, and RD-ES (Ewing sarcoma) cell lines were tested for the EWS-FLI1 fusion transcript. When RT-PCR was performed using the primer set 11.4 FLI1 and 22.4 EWS (see “Materials and Methods”), two products (130 and 250 bp) were detected in the 231-EIA and HEK 293 cells but not in the parental MDA-MB-231 or vector-transfected control cells. However, the EWS-FLI1 probe did not hybridize to these products on Southern hybridization (Fig. 1a), indicating that they do not represent the EWS-FLI1 fusion transcript. The same membrane was then hybridized with the radiolabeled 22.4 EWS oligonucleotide primer, confirming transfer of the DNA visualized on the ethidium-stained gel to the membrane. The amplification pattern obtained when nested PCR was performed using the external 11.3 FLI1 and 22.3 EWS primers followed by the nested internal 11.4 FLI1 and 22.4 EWS primers was the same as that obtained after PCR with 11.4 FLI1 and 22.4 EWS (described above; data not shown). RT-PCR with the 11.3 FLI1 and 22.4 EWS primers amplified a 340-bp product in 231-EIA cells but not in the parental cells or vector controls. Again, Southern blotting was negative for these samples (Fig. 1b). Thus, although E1A is able to induce transcripts that can be amplified by primers derived from the EWS and FLI1 genes, the E1A-induced transcripts do not represent the EWS-FLI1 fusion transcripts.

To confirm and understand what may cause false positive RT-PCR results in E1A-transfected cell lines, the RT-PCR products amplified from the 231-EIA cell line and the Ewing sarcoma line RD-ES were subcloned and sequenced. Sequencing of the RT-PCR product amplified from the RD-ES cell line with the 11.4 FLI1 and 22.4 EWS primers confirmed that it was the EWS-FLI1 fusion product (Fig. 1c). In contrast, sequencing of the bands from the 231-EIA transfectants (Fig. 1a) proved them to be unrelated genes; the 250-bp band corresponded to the mRNA for the human KIAA0477 protein, and the 130-bp band corresponded to the mRNA for the human KIAA0339 protein. The RT-PCR products were homologous to the fusion product EWS-FLI1 only in the primer sequences, with 100% homology in the 3' ends of the 11.4 FLI1 and 22.4 EWS primers (Fig. 1d). The sequence of the 340-bp product amplified from the 231-EIA cell line with 11.3 FLI1 and 22.4 EWS primers (Fig. 1b) corresponded to that of human ribosomal protein L4 mRNA. Again, there was sequence homology between the 22.4 EWS primer and the L4 sequence (the L4 3' untranslated region sequence annealing to the 11.3 primer is not available in GenBank for comparison). Thus, all of the amplified RT-PCR products in cell lines other than RD-ES, represented false positive results. These genes may be up-regulated with E1A transfection and deserve further study.

A total of 13 stable E1A transfectant E1A cell lines including MDA-MB-231, MCF-7, MDA-MB-435 (breast cancer), SKOV3-ip1 (ovarian cancer), and PC-3 (prostate cancer) were tested for the EWS-FLI1 fusion transcript using RT-PCR. The actual EWS-FLI1 fusion transcript could not be detected in any of the E1A transfectant E1A cell lines.
of the cell lines except the RD-ES Ewing sarcoma line. RT-PCR was also performed with primers specific for E1A, and expression of the E1A transcript was confirmed in the E1A-transfected cell lines (data not shown). The E1A transcript was not detected by RT-PCR in the RD-ES Ewing sarcoma cell line (Fig. 1e). Thus, our study does not support the proposal by Sanchez-Prieta et al. (5) that E1A induces the Ewing tumor fusion transcript EWS-FLI1.

Fig. 1  RT-PCR and Southern blot hybridization of the fusion transcript EWS-FLI1.  

a, top panel, RT-PCR analysis with primers 11.4 FLI1 and 22.4 EWS on total RNA from MDA-MB-231 cells, vector-transfected 231 cells, E1A-transfected 231 cells, HEK 293 cells, and RD-ES Ewing sarcoma cells. Upper middle panel, RT-PCR of the same cell lines with primers for GAPDH as a control. Lower middle panel, Southern blot analysis of RT-PCR products shown in the upper panel with a EWS-FLI1 fusion product probe. Bottom panel, Southern blot of the same membrane with radiolabeled 22.4 EWS oligonucleotide probe as transfer control.  
b, top panel, RT-PCR analysis of RNA from the same cell lines with primers 11.3 FLI1 and 22.4 EWS. Middle panel, RT-PCR for GAPDH as a control. Bottom panel, Southern hybridization of RT-PCR products with the EWS-FLI1 probe.  
c, sequence corresponding to the EWS-FLI1 fusion transcript detected in the RD-ES cell line. The arrow indicates the breakpoint. RT-PCR using primers 22.4 EWS and 11.4 FLI1 would be expected to give a 344-bp product as detected in a; primers 22.4 EWS and 11.3 FLI1 would be expected to give a 363-bp product as detected in b.  
d, sequence alignment of the 22.4 EWS and 11.4 FLI1 primers with human mRNA for KIAA0477 protein (dbj, AB007946.1), human mRNA for KIAA0339 gene (dbj, AB002337), and human ribosomal protein L4 (RPL4) mRNA (ref, NM 000986.1). Sequences with homology to the primers are underlined. The numbers in parentheses in the figure represent the location on the reference sequence used for alignment.  
e, RT-PCR analysis of RNA from 231-E1A and RD-ES cell lines with E1A primers.
E1A-transfected Cells Do Not Express the EWS-FLI1 Fusion Protein. The human breast cancer cell lines MCF-7 and MDA-MB-231 and ovarian cancer cell line SKOV3-ip1 were studied with and without transfection of E1A to detect the EWS-FLI1 fusion protein. The Ewing sarcoma cell line RD-ES was used as a control for the EWS-FLI1 fusion protein. After immunoblotting with a polyclonal antibody against EWS, a $M_r \sim 68,000$ band was detected in all cell lines studied except SKOV3-ip1 and E1A-transfected ip1-E1A cells (Fig. 2). When a monoclonal anti-FLI1 antibody was used, the expected $M_r 68,000$ EWS-FLI1 fusion protein was detected only in the RD-ES cell line (Fig. 2). No fusion proteins were detected in any of the E1A-transfected cell lines. Immunoblotting with E1A antibodies confirmed the expression of the E1A protein in the E1A-transfected cell lines. The results indicate that the commercially available anti-EWS polyclonal antibody (Santa Cruz Biotechnology) also cross-reacts with a cellular protein about the same size ($M_r \sim 68,000$) as the EWS-FLI1 fusion protein. However, the $M_r \sim 68,000$ band detected in HEK 293, MCF-7, MCF-7-E1A, 231, and 231-E1A cells is not the EWS-FLI1 protein because it cannot be detected by the anti-FLI1 antibody.

Discussion

Sanchez-Prieto et al. (5) recently reported that adenovirus E1A induces the Ewing sarcoma fusion transcript EWS-FLI1. However, data from the studies of Melot and Delattre (6) and from Kovar et al. (7, 8) do not support this hypothesis. In our study, we examined 13 different cell lines stably transfected with E1A to test the hypothesis that expression of the E1A gene in human cells could elicit the specific fusion transcript EWS-FLI1. We were unable to find any evidence of the EWS-FLI1 fusion transcript or fusion protein in these E1A transfecants. Interestingly, we found expression of unrelated genes that may be induced by E1A, which can be detected under the PCR assay conditions used (Fig. 1, a and b). In addition, the commercially available anti-EWS antibody also cross-reacts with a cellular protein about the same size ($M_r \sim 68,000$) as the EWS-FLI1 fusion protein (Fig. 2). It is worthwhile to mention that the anti-EWS antibody-detected $M_r \sim 68,000$ protein is slightly enhanced in the E1A-transfected cells (compare MCF-7 with MCF7-E1A and 231 with 231-E1A). This phenomenon, without careful further examination with anti-FLI1 antibody to exclude its identity as the EWS-FLI1 fusion protein, could be easily but mistakenly used to support the notion that E1A induces the EWS-FLI1 fusion protein.

Adenovirus E1A has been examined in preclinical studies and found to be a promising therapeutic agent because of its abilities to suppress tumorigenesis and metastasis and to sensitize cells to apoptosis induced by cytotoxic agents (10–16). Extensive preclinical safety studies have been performed with E1A (17–19), and a Phase I clinical trial of E1A gene therapy for patients with advanced breast and ovarian cancer has recently been completed (3). Phase II studies of E1A gene therapy are currently in progress.

The proposal that E1A may induce EWS-FLI1 expression is revolutionary because it suggests that the Ewing tumor may be of viral etiology and that viral transfections may lead to chromosomal translocations. This hypothesis has implications regarding the safety of the use not only of E1A but also of adenoviral vectors containing E1A, such as Onyx-015 (20) in gene therapy trials. Our findings, however, support those of Melot and Delattre (6), and Kovar et al. (7, 8); we found no evidence for an association between E1A expression and expression of the EWS-FLI1 fusion product.

We have not studied the E1A integration site in the genome of our stable E1A transfectants. The integration site may be important for the activation of genes; however, Sanchez-Prieto et al. (5) have found that E1A-expressing HEK 293 and IMR 90 cells have multiple gene copies in many chromosomes, with no specific chromosomal site of integration. Thus, the site of gene integration is unlikely to account for differences in our results. Although we cannot exclude the possibility that some E1A-expressing cell lines may have the EWS-FLI1 fusion as claimed by Sanchez-Prieto et al. (5, 9), the presence of such a fusion is not characteristic of E1A-transfected cells. We conclude that the potential for induction of such a gene rearrangement using E1A gene therapy is too low to be of clinical significance in the treatment of advanced malignant disease.

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References


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